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**Review Article**

# ISCOMs (immunostimulating complexes): The first decade

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**Summary** A little over a decade ago, novel immunostimulating complexes (ISCOMs) were described. This review examines the position and progress that ISCOM technology has achieved in the fields of vaccine research and medicine over this period. Much of the work on ISCOMs has remained in the area of vaccine research where there is still an urgent need for improved adjuvants to help combat important diseases such as AIDS, malaria and influenza. Currently the only widely licensed adjuvants for human use are the aluminium salts, but with the trend towards highly purified subunit vaccines, which are inherently less immunogenic than some of the older vaccines, potent adjuvants capable of promoting specific immune responses are required. ISCOMs are one such technology that offers many of these requirements and as their use in vaccines enters its second decade clinical trials are commencing that will establish whether these submicron, non-living particles composed of saponin, cholesterol, phospholipid and in many cases protein, are useful components for a range of human vaccines.

**Key words:** adjuvants, immune response, immunization, ISCOMs, vaccine.

## Introduction

The acronym ISCOMs was derived from the ability of these submicron particles to act as immunostimulating complexes in animals when used in vaccines, initially prepared from membrane proteins of parainfluenza-3, measles and rabies viruses.[1] This property of ISCOMs as a vaccine adjuvant, remains the chief focus of research and commercial development of the technology to date. The term adjuvant is used in many ways in biology and medicine. Here it will be used to refer to compounds which act in a non-specific manner to enhance specific immunity to an antigen.[2]

The history of vaccine adjuvant research is littered with numerous examples of heralded new adjuvants that have not lived up to expectations or have a more restricted utility than was first claimed. Much basic research on adjuvant development has been undertaken within the bio-pharmaceutical industry: thus a considerable amount of information on adjuvants is not in the public domain or readily available. Vaccine manufacturers would like to move away from aluminium-based adjuvants which predominantly induce a limited repertoire of circulating antibodies to adjuvants that provide a broader immune response. In addition, increasing use of highly purified proteins derived by recombinant DNA technology and synthetic antigens, most of which are poorly immunogenic, has increased the demand for alternative potent and predictable adjuvants.

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Why have adjuvants remained an unattractive area of investigation for large, well-funded research groups? One major deterrent has been the general inconsistency of adjuvants. Frequently, a group of adjuvants has been ranked for efficacy with a particular antigen in a particular experimental animal only to have the order reversed with a change in animal species or antigen. Thus, unifying hypotheses have been difficult to formulate. This has led to a situation in many research laboratories where a monumental effort is expended on producing, purifying and comprehensively characterizing an immunogen only to mix it prior to injection with an adjuvant, about which little is known, except that it is easy to formulate. One then hopes for the best in terms of immunity or protection following challenge!

The enormous research effort in the study of immunoregulation over the past 25 years has led to a detailed definition of lymphocyte populations, the elucidation of the nature of MHC-restricted antigen recognition and, above all else, the identification of cytokines. As a result, adjuvant research, which was largely whole-animal oriented, can now make use of a wide range of tools for the dissection of cellular and subcellular events. Thus, it will be possible to precisely define what an adjuvant can or cannot do and clearly elucidate its primary mode of action.

Despite the substantial strides taken in recent times, aluminium salts are still the only artificial adjuvant widely licensed for humans and none of the newer adjuvants have been registered for human use, although several are in advanced clinical trials. One of these, ISCOMs, emerged in the early 1980s and after a slow start, probably

due to perceived difficulties with formulation, is now regarded as an extremely promising adjuvant for both human and animal use. The unique characteristic of ISCOMs appears to be induction of a wide range of protective immune mechanisms including MHC class I-restricted CTL responses, high levels of specific, neutralizing antibody and secretion of various cytokines.

## Definition of an ISCOM

The terms ISCOMS and ISCOM are commercial trademarks of Iscotec AB, Sweden; but the more generic term ISCOMs will be used throughout this article. Two types of ISCOMs have been described in the literature. The classic ISCOMs formed from cholesterol, saponin, phospholipid and viral envelope proteins, and the basic ISCOM which is formed in the absence of envelope proteins and is termed ISCOM matrix or empty ISCOMs (ISCOMATRIX is a commercial trademark of Iscotec AB). Figure 1 shows electron micrographs of ISCOM matrix, influenza-ISCOMs and purified influenza viral particles for comparison. Typically ISCOMs and ISCOM matrix particles are hollow, spherical, cage-like particles that have a heterogeneous size distribution of around 40 nm in diameter and are negatively charged.[1,3–5] An individual ISCOM or ISCOM matrix particle is a collection of some 20 or more subunits (see Fig. 1) which when formed together are extremely stable to prolonged storage and freeze-drying.[6,7] When measured by density gradient centrifugation, ISCOMs and ISCOM matrix exhibit a sedimentation rate of 14–19S depending on their lipid content[8] and both forms have been shown to enhance immune responses to a number of candidate vaccine molecules. A more liberal definition of the term ISCOM was suggested by Kersten[6] as 'all saponin–lipid complexes prepared for use as an adjuvant or an antigen vehicle'; however, this definition has not been generally adopted.

## A brief history of ISCOMs

ISCOM-like structures were first observed in 1971 in virus preparations treated with saponin while isolating viral subunits but were regarded as artifacts.[9] Morein *et al.*[1] were the first to describe a method for incorporating viral membrane components into saponin-containing complexes to produce the well defined, highly immunogenic particles from which the term ISCOMs was coined. Since publication in 1984, approximately 180 scientific papers have been published (other reviews[4,10–14]). The original idea behind ISCOMs was to create a particle on to which several copies of a protein, peptide or other molecules could be attached which could then be used to promote an enhanced immune response. This has now been demonstrated for a variety of human and animal pathogens. A list of some of the important stages in the development of ISCOM technology is given in Table 1, although the crucial step, registration of an ISCOM-based vaccine for use in humans, has yet to be accomplished. Since their inception, ISCOMs have certainly fulfilled Morein's original prediction that 'the ISCOM might be an interesting new form of a subunit vaccine'.[1]

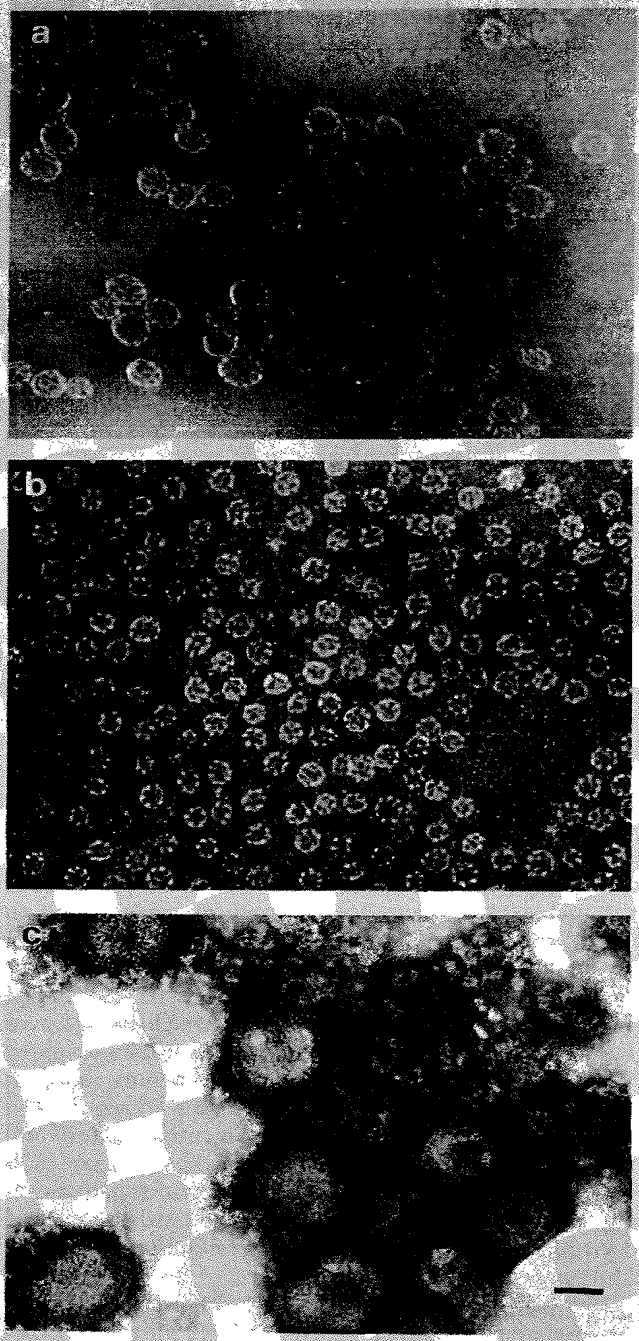


**Figure 1** Electron micrographs of (a) ISCOM matrix, note the characteristic 'soccer ball' shape of the particles, (b) influenza ISCOMs with prominent HA 'spikes' on the surface and (c) purified influenza virus. The bar represents 50 nm.

## The basic structure and composition of ISCOMs

### *The ISCOM matrix composition*

The basis of the unique structure of ISCOMs is the interaction of saponin and cholesterol. Saponins are some of the most common secondary constituents of plants and

**Table 1** Milestones in the development of ISCOMs

| Year | Milestone |
|---|---|
| 1984 | Morein and co-workers first to describe and publish on ISCOMs[1] |
| 1985 | Induction of a protective response in cats with feline leukaemia virus ISCOMs[122] |
| 1987 | Preformed ISCOMs used as a carrier for a peptide vaccine[52] |
| 1988 | Basic requirements (cholesterol, phospholipid, Quil A) for consistent production of ISCOMs established[3,8] |
| | Purification of Quil A saponins by reversed phase HPLC[3,123] demonstated as a way to obtain less toxic saponin species[123] |
| | First US ISCOM patent granted |
| | First ISCOM-based vaccine sold by Iscotec AB (equine influenza vaccine for horses) |
| 1990 | First demonstration of ISCOMs inducing a $CD8^+$ MHC-I-restricted CTL (in HIV-1)[83] |
| 1991 | One hundredth publication on ISCOMs achieved |
| 1994 | Preclinical toxicology on ISCOMs commenced |
| 1995 | Clinical trials with ISCOM matrix commenced |
| 1995 | First human Phase I clinical trials with an influenza ISCOM-based vaccine |

are found in more than 500 plant genera. Their physiological function is not clearly understood but they may contribute to the depot nourishment of the plant by the release of monosaccharides and they may also exert an anti-microbial activity. Saponins are found in all parts of the plant but in the *Quillaia* (also written as *Quillaja*) *saponaria* tree, the highest content is in the bark. Crude saponin extracts from bark have been widely used in applications such as film emulsions, as a mild detergent in shampoo and as a foaming agent in beverages. The use of saponins in immunological applications had its beginnings in the 1920s when a variety of substances were tested for adjuvant activity with formalin-toxoided vaccines.[15] However, except in experimental vaccines, they were not used extensively until Espinet[16] reported on the value of saponin, available in Argentina, in foot and mouth disease vaccines (FMD). Several years later Dalsgaard[17] demonstrated that of the commercially available saponins being used in FMD in the 1960s, only those derived from the South American tree *Quillaia saponaria* Molina were effective.

Using an aqueous extract of bark followed by dialysis, ion exchange chromatography and gel filtration, Dalsgaard[18] identified the active fraction from *Quillaia saponaria* Molina bark. This fraction, which he termed 'Quil A', represented only 1% by weight of the bark and was found to be a single band on TLC. Furthermore, only one-fifth of the dose was required to achieve the same immunogenicity as crude saponins in FMD, with a subsequently lower incidence of site reactions.[19,20] By incorporating Quil A into ISCOMs, even lower levels of Quil A were administered, which further reduced site reactions.[4] Quil A has been the major saponin used for ISCOM work to date. It has usually been obtained from commercial sources such as Superfos (Vedbaeck, Denmark), Accurate Chemical and Scientific Corporation (Westbury, NY, USA) or Iscotec AB (Uppsala, Sweden) where it is sold under the trade name 'Spikoside'.

While Quil A appeared homogeneous in earlier TLC studies,[17] modern techniques such as reversed phase HPLC and high performance TLC revealed that the material contained several components.[3,21–23] Twenty-two separate major peaks that varied in their adjuvant activity, toxicity, haemolytic activity, molecular weight and monosaccharide composition were distinguished in samples of Quil A.[23] These Quil A peaks were analysed and the different properties and characteristics of the fractions were shown to correlate with differences in their monosaccharide composition.[23] QS-21[24] a fraction that was identified during this work is now undergoing intensive testing as a candidate vaccine adjuvant. The first structure of Quil A, QS111, was defined by Higuchi *et al.*[22] as a triterpene glycoside with two branched sugar chains at positions 3 and 28 of the aglycone (the ring structure).

Limited studies have examined other sources of saponin for use in ISCOMs. Bomford *et al.*[25] examined several sources and demonstrated adjuvant activity when mixed with keyhole limpets haemocyanin (KLH) and injected into mice. Only saponins that were structurally closely related to *Quillaia* saponins such as *Gypsophila* and *Saponaria* saponins had adjuvant activity. These saponins were also examined for their ability to form ISCOM matrix particles. Typical discrete particles were formed only when *Quillaia saponaria* saponins were used, while the saponins from the other sources formed large complexes or lattice-like sheets. No immunogenicity was reported using ISCOMs made from these other saponins.

As indicated previously, cholesterol is an essential component of ISCOMs and ISCOM matrix. Saponins have long been known to interact with lipids in cell membranes forming complexes that lead to cell lysis.[26–29] When a series of lipids (cholesterol, dipalmitoyl phosphatidylcholine, palmitic acid, myristic acid, phosphatidylethanolamine, dipalmitoyl phosphatidylserine, phosphatidylglycerol, sphingomyelin or cerebroside) were tested for their ability to combine with Quil A to form ISCOM matrix, only cholesterol was effective.[8] Studies by Kersten *et al.*[3,6] using slight variations on this method found that the addition of phospholipid was also required to form the typical ISCOM matrix and if phospholipid was absent, ring-like structures or sheet-like lamellae were formed depending on the ratio of Quil A to cholesterol (w/w). Özel *et al.*[5] also noted that ISCOM matrix particles formed without phospholipid were fragile, slightly flattened cage-like structures. It appears that while the inclusion of phospholipid may not always be critical for forming ISCOM matrix, addition of phospholipid gives more uniform, reproducible ISCOM matrix particles which may also be more stable. Furthermore, as discussed in the next section, phospholipid is essential when ISCOMs containing proteins are to be made.

### *The role of phospholipid*

The requirement of phospholipid in ISCOMs containing amphipathic molecules was not recognized in the initial

work as phospholipid was coextracted from the virus or bacterium preparations (and thus available for incorporation into ISCOMs) during solubilization with non-ionic detergents.[1,11] However, when attempts were made to incorporate highly purified membrane proteins into ISCOMs, mixed micelles or aggregates were instead formed.[8] This problem was overcome by the addition of phospholipids such as phosphatidylcholine[8] or phosphatidylethanolamine.[3] Phospholipid allows amphipathic molecules to insert into ISCOMs,[14,30] perhaps by providing a looser 'fit' than cholesterol alone.

### *The ISCOM structure*

The composition by weight of an ISCOM is approximately 10–15% cholesterol and phospholipid, 60–70% saponin and 10–15% protein.[3,31] The arrangement of the cholesterol–saponin subunits into the ISCOM particle has been investigated by Özel *et al.*[5] who proposed a pentagonal dodecahedron arrangement of approximately 20 smaller 12 nm subunits which when aggregated together made up a single ISCOM particle of approximately 40 nm in diameter. Kersten *et al.*[31] proposed a slightly different arrangement of the subunits suggesting that they were held together by hydrophobic interactions, steric factors and possibly hydrogen bonds. Freeze-fracture electron microscopy has shown ISCOMs to be hollow, spherical structures with surfaces that show small pore like intrusions.[3] Other features of the ISCOM structure noted by Kersten *et al.*[31] were their rigid nature (comparable with the most rigid liposomes they had studied), negative surface potential at physiological pH (which prevents aggregation), and the presence of a hydrophilic core in the subunits through which small water soluble molecules could diffuse.

### *Recent modifications to the basic components*

*Quil A component* The major modification to ISCOM technology has been the use of fractionated *Quillaia saponaria* Molina saponins. From Quil A, Kensil *et al.*[23] purified milligram quantities of the four major components and designated them QS-7, QS-17, QS-18 and QS-21. Interestingly, QS-7 and QS-21 had lower toxicity than Quil A or QS-18 as determined by lethality studies in CD-1 mice.[23] Iscotec AB have also developed similar procedures for fractionating crude saponins by reversed phase HPLC but have selected three fractions termed QH-A, QH-B and QH-C according to their degree of hydrophobicity (QH-A < QH-B < QH-C).[32] Ronnberg *et al.*[32] have examined the characteristics of these QH fractions and ISCOMs made from these fractions. Extensive haemolysis of chicken RBC *in vitro* was obtained when free QH-A, QH-B, or QH-C was used, but when the QH fractions or mixtures of QH-A, QH-B, QH-C (e.g. 3:6:1 w/w) were incorporated into ISCOM matrix, no haemolysis was seen at levels of up to 100 μg/mL. Lethality studies in ICR mice showed higher toxicity associated with QH-B (7/10 deaths at 400 μg) than with QH-A or QH-C (0/10 deaths at 400 μg for both) or when QH-C was incorporated into ISCOM matrix (0/10 deaths at 800 μg).[32] A combination of 7 parts QH-A with 0 parts QH-B and 3 parts QH-C (known as ISCOPREP 703), which approximates the natural ratios of QH-A:QH-C found in crude *Quillaia* saponin, has recently been extensively tested as a component of a new ISCOM-adjuvanted human influenza vaccine.

*Lipids* While cholesterol is essential for ISCOM formation there is some flexibility in the choice of the phospholipid. Recent studies in the our laboratories examined 15 phospholipids and found that practically all formed ISCOMs with purified influenza virus. Further work aims to examine the *in vitro* haemolytic activity, toxicity and immunogenicity of ISCOMs and ISCOM matrix made with different phospholipids.

## ISCOMs containing amphipathic molecules

ISCOMs have been made with amphipathic molecules derived from cell walls and membranes from a variety of viruses, bacteria and parasites.[12] including HSV-1, CMV, EBV, HBV, rabies, influenza, *Escherichia coli*, *Brucella abortus*, *Toxoplasma gondii* and *Plasmodium falciparum*. When ISCOMs include antigens derived from purified organisms or cell membranes, the molecules that are incorporated into ISCOMs are proteins or glycoproteins which are normally anchored by a hydrophobic transmembrane sequence into the cell or viral membrane.[14] These molecules are extracted by detergent treatment and are integrated into the lipid–*Quillaia* saponin matrix when the detergent is removed and the ISCOMs are formed.[31,33] The manner in which these molecules integrate into ISCOM has been studied by Kersten *et al.*[3] with the major outer membrane pore protein (porin or PI) of *Neisseria gonorrhoea*. By analysing enzyme and cyanogen bromide cleavage fragments of PI obtained from bacterial membranes, ISCOMs with PI incorporated, or from purified PI, they found that the orientation of the PI in ISCOMs was similar but not identical to its orientation in the outer membrane of the bacterium. They also showed that the amphiphilicity of the molecule could affect the incorporation of the PI suggesting that the balance of hydrophobic and hydrophilic regions is important and that the presence of a hydrophobic transmembrane sequence alone is insufficient for insertion of molecules into ISCOMs. One limitation of this study is that this PI molecule is a somewhat atypical molecule with an uncommon arrangement of three antiparallel β-sheets that form the barrel-like channels through the bacterial outer membrane.[34]

Single and multiple amphipathic molecules have been incorporated into ISCOMs;[3,35–41] regardless of the type of molecules, the size, level of glycosylation or tertiary structure, ISCOMs are all similar and usually indistinguishable from ISCOM matrix when examined by electron microscopy or sedimentation rate.[4,5,12,31,42] This is somewhat surprising if a substantial amount of protein is incorporated into ISCOMs, but may be explainable by inter-

actions between the surface of the ISCOM and the protein, the partial entry of the molecule into the ISCOM or may simply reflect the low number of molecules incorporated per ISCOM particle. Support for this latter point is the observation that when high levels of influenza haemagglutinin (HA) are incorporated into ISCOMs, 'spikes' of HA can be visualized on the surface of the ISCOMs by electron microscopy (Fig. 1).

## ISCOMs containing non-amphipathic molecules

While amphipathic membrane proteins readily integrate into ISCOMs, the efficiency of incorporation of soluble, hydrophilic proteins or very hydrophobic proteins into ISCOM is low.[10,43] Unlike liposomes where there is entrapment of these types of proteins, ISCOMs because of their very small size and internal volume are unable to encase proteins in this manner. A number of methods have therefore been investigated to expose or introduce hydrophobic regions into non-amphipathic peptides and hydrophilic proteins so that these molecules can be linked to ISCOMs. Whether these molecules are inserted into ISCOMs in the same manner as truly amphipathic molecules or merely associated with the ISCOM matrix remains to be determined but probably varies according to the protein and method of association. The simplest methods have involved exposing the protein to pH 2.5 buffers with Pyle *et al.*[38] and Morein *et al.*[43] obtaining 15 and 14% incorporation of purified HIV-1B gp120 and BSA, respectively, into ISCOMs. Heeg *et al.*[44] also claimed good (but not quantitated) incorporation of ovalbumin into ISCOMs with the same treatment. ISCOMs formed by this method, which contained the partially denatured BSA and HIV proteins, stimulated better antibody responses in mice and rhesus monkeys than the unincorporated protein alone.[38] The antiserum raised against the gp120 was also functionally active in limited testing for *in vitro* HIV-1 viral neutralization.[38,43] Heeg *et al.*[44] also showed high levels of MHC class I-restricted CTL in the draining lymph nodes of mice injected with ovalbumin–ISCOMs that were not present in the lymph nodes of mice receiving ovalbumin alone. No comparisons, however, were reported with controls where the protein was administered after mixing of ISCOM matrix or Quil A with ovalbumin. Higher levels of incorporation may be possible with these denaturation techniques, as Morein *et al*[43] has claimed 15–80% incorporation of purified rabbit or mouse immunoglobulin after acid treatment and Hoglund *et al.*[4] has reported that treatment at 70°C resulted in incorporation of 30–50% of BSA. It is likely that incorporation (rather than association) is required for the efficient induction of CTL but this has yet to be proven conclusively.

Alternatively, partial denaturation, using agents such as urea and mercaptoethanol,[40] have been used in an attempt to uncover some of the hydrophobic regions within proteins. Treatment of recombinant HIV proteins, including a *gag* fusion protein, p24-p15 and PB1 (the carboxy-terminal portion of gp120), allowed incorporation of approximately 20% of the starting material for each of the proteins into ISCOMs after denaturation with 8 mol/L urea and 15 mmol/L mercaptoethanol, but this was far below the 80% incorporation of another recombinant HIV protein gp160 which has a native transmembrane anchor and did not require partial denaturation.[49]

The methods described above, however, may lead to a loss of conformationally-important determinants. Another strategy is to covalently attach fatty acids to the protein and several proteins such as ovalbumin, cytochrome C, Tamm Horsfall glycoprotein and HIV-1 IIIB gp120 have been successfully incorporated into ISCOMs by covalent attachment of palmitic acid through the ε-amino groups on lysines in the protein.[45–48] While it is possible that the attachment of palmitic acid will alter individual epitopes, this has not been described to date. Kanzanji *et al.*[48] reported 33–64% incorporation of palmitified p27 (a purified recombinant surface protein from the *Eimeria falciformis* sporozoite) into ISCOMs over a number of experiments. Mice given the palmitified p27-ISCOMs orally showed improved pre- and post-challenge responses compared with mice administered palmitified p27 alone, by the same route. Mowat *et al.*[45] reported similar results with palmitified ovalbumin incorporated into ISCOMs. This material was capable of inducing delayed-type hypersensitivity (DTH) responses in mice when injected into the footpad, unlike ovalbumin, palmitified ovalbumin or palmitified ovalbumin injected with Quil A or ISCOM matrix. Furthermore, specific CTL which lysed target cells could be detected in the spleens of mice given ISCOMs containing palmitified ovalbumin orally, but not in the spleens of mice fed ovalbumin alone. No data were reported on CTL derived from the spleens of mice administered palmitified ovalbumin alone or palmitified ovalbumin combined with Quil A or ISCOM matrix. In a later publication Reid[46] stated that Mowat had found none of these formulations capable of inducing CTL in this system and that only by incorporating the palmitified ovalbumin into ISCOMs were they able to generate CTL after oral dosing.

A novel method to associate influenza nucleoprotein with ISCOMs was described by Weiss *et al.*[49] Chemical coupling (by the periodate oxidation method) was used to link influenza nucleoprotein to *E. coli* LPS which was then incorporated into ISCOMs. This allowed association of 10–20% of the nucleoprotein with ISCOMs. Immunization of mice with the resultant material gave partial protection from challenge with the same influenza strain and a high antibody response, whereas BSA coupled to the LPS in the same manner and formed into ISCOMs did not protect mice or induce influenza-specific antibody. The problem of incorporating non-amphipathic molecules into ISCOMs has also been approached by utilizing genetic manipulation to re-engineer the protein to contain a transmembrane sequence. Rimmelzwaan *et al.*[41] modified the *env* gene of feline immunodeficiency virus by removing a cleavage site that resulted in the expression of a 150 kDa protein with an intact transmembrane domain. The 150 kDa protein, when incorporated into ISCOMs, induced higher antibody responses in cats than did the native 130 kDa protein given alone or when combined with Quil A.

The simplest method for incorporating a peptide into ISCOMs involves the addition of fatty acid tails such as palmitic acid[50] or myristic acid[51] during peptide synthesis. Pedersen *et al.*[50] reported approximately 40% incorporation of a series of palmitified peptides into ISCOMs, which when administered to rabbits stimulated good antibody levels to some peptides but lower responses compared with previous studies in which the peptides were linked to KLH. They speculated that these differences may be due to the lack of appropriate T-helper cell epitopes in some of the peptides. Weijer *et al.*[51] showed better immunogenicity in cats and rabbits with myristillated peptides incorporated into ISCOMs than achieved by simply mixing the peptides with ISCOM matrix or given as peptides adsorbed to $Al(OH)_3$. There may be size and sequence limitations on particular peptides as some short, poorly-charged peptides may be too hydrophobic or may lack the right hydrophobic–hydrophilic balance for incorporation into ISCOMs.[13] Peptides from 10–40 residues have been successfully modified with lipid tails and incorporated into ISCOMs.

Another method used to associate molecules with ISCOMs has been to chemically couple peptides and proteins on to preformed ISCOM matrix or ISCOM containing influenza envelope proteins.[52–55] When coupling is via influenza envelope proteins, conventional coupling agents such as glutaraldehyde and heterobifunctional reagents have been used.[52,55] Potentially these techniques make it possible for multiple antigens to be attached to the same ISCOM particle and immune responses have been generated not only to the coupled antigens but also to the influenza envelope protein to which it is linked.[53] Lövgren *et al.*[52] have shown that, with the coupling of biotin to preformed influenza–ISCOM, conjugation of at least three biotin molecules per viral protein in the ISCOMs was required for good immunogenicity, 10 biotin molecules per viral protein being the optimal epitope density. Coupling to ISCOM matrix alone can also be achieved by replacing the phosphatidylcholine with phosphatidylethanolamine and using the amino group for covalent linking, although results using this technique have not yet been published.[13]

## Preparation of ISCOMs

Methods for the small scale production of ISCOMs have been described in several previous publications[1,4,8,12,56] and a summary of the steps involved is shown in Table 2. Of the two methods that have been used extensively, dialysis has gained favour over centrifugation because of its simplicity and greater potential for scaling up. An important factor in formation of optimal ISCOMs is the ratio of the various components which are mixed together at the start. The optimal weight ratios of cholesterol:phospholipid:*Quillaia* saponin to obtain typical ISCOM matrix was found to be 1:1:5; while ratios of 1:1:5:0.1 and 1:1:5:1 cholesterol:phospholipid:*Quillaia* saponin:protein have been reported for various amphipathic proteins incorporated into ISCOMs.[8,31]

The choice of detergent for solubilization of membranes and lipids may be important for particular epitopes. Merza *et al.*[56] reported a differential recognition of epitopes with monoclonal antibodies after the production of ISCOMs containing the bovine leukaemia virus gp51 with several different non-ionic detergents. Surprisingly they found *n*-octylglucoside, Tween 20 or Tween 80 preserved the conformation of gp51, while MEGA-10 and Triton-X 100 did not. This may not be an isolated observation peculiar to this protein as there have been other

**Table 2** Preparation of ISCOM (for amphipathic molecules only)

1. Purify and concentrate the virus, bacterium, parasite or protein of interest.

2. Solubilize the membrane proteins or purified protein using a detergent, usually a non-ionic detergent such as Triton X-100, MEGA-10 or *n*-octylglucoside. A non-ionic detergent is preferable as its interaction is limited to the hydrophobic regions of the molecule and there is little or no effect on the hydrophilic parts of the molecule (these areas may be important in inducing protective immunity and should therefore not be modified).[4]

3. Remove insoluble material/proteins by centrifugation

4. Formation of ISCOMs by one of two methods

| Centrifugation method | Dialysis method |
|---|---|
| 4a. Solubilized proteins are spun through a thin layer of 10% sucrose containing 0.5% of a non-ionic detergent e.g. Triton X-100, which is layered on top of a 10–40% sucrose gradient containing 0.1–0.2% Quil A and spun in an ultracentrifuge. | 4b. Solubilized proteins are added to cholesterol and phospholipid dissolved in a non-ionic detergent e.g. MEGA-10 (or another detergent with a high CMC for easy removal by dialysis) to which Quil A or a purified saponin is added and mixed. |
| 5a. Fractions from the sucrose gradient around the 19S value are removed and pooled. | 5b. Detergent is removed from the mixture by extensive dialysis or ultrafiltration with a number of buffer changes. |
| 6a. Free Quil A is removed by ultracentrifugation through a 10% sucrose gradient cushion to pellet ISCOMs. | 6b. (Optional) Removal of components not included into ISCOMs (Quil A or protein micelles) by ultracentrifugation through a 10% sucrose gradient. ISCOMs are pelleted. |
| 7a. Dialyse to remove sucrose. | 7b. (Only if 6b is done.) Dialyse to remove sucrose. |

8. Analysis of ISCOM formation and characteristics such as; morphology (by negative contrast electron microscopy), size (by electron microscopy or particle sizer), protein assay and content (by SDS-PAGE analysis), immunogenicity and antigen content (by ELISA and western blotting), sedimentation coefficient (by ultracentrifugation), Quil A content (by HPLC).

cases of denaturation with these detergents in ISCOM work (Bror Morein pers. comm.). The majority (61/71) of publications on ISCOMs have used non-ionic detergents with MEGA-10[57] (deconyl-*N*-methyl glucamide) being the most favoured (30/71) followed by Triton X-100,[1] (21/71), and *n*-octylglucoside[58] (8/71) with occasional reports on the use of other non-ionic detergents such as MESK[59] and MEGA-9.[60] The ionic detergent sodium deoxycholate[45,46] has been used when incorporating palmitified proteins into ISCOMs and the zwitterionic detergent Empigen BB[61,62] has also been used for producing HSV ISCOMs. One potential problem in the preparation of ISCOMs is the incorporation of unwanted molecules especially when whole viruses are used. For example, when cell culture-derived HIV-1 virus was purified, solubilized and incorporated into ISCOMs, the major constituent was found to be HLA-DR which was derived from the host cell and forms part of the viral envelope.[63] When administered to mice, rabbits or guinea-pigs these ISCOMs induced high titres of antibody to the HLA-DR molecules and low levels of antibody to the target molecule, HIV-1 envelope glycoprotein gp120, which was the minor protein species incorporated into the particle. Removal of unwanted amphipathic molecules prior to the formation of ISCOMs has been achieved by immunoabsorbent chromatography using immobilized, specific mAb.[64]

There have been no publications on large scale production of ISCOMs, reflecting the lack of commercial products utilizing this technology. Iscotec AB have manufactured an ISCOM-based equine influenza vaccine for horses in Europe for a number of years. This vaccine is made from two egg-grown strains of equine influenza with the viruses being purified, disrupted with Triton X-100 and formulated into ISCOMs with MEGA 10 followed by ultrafiltration to remove the detergent. Approximately 20 000 doses of the ISCOM-vaccine are made per batch.

## Mode of action of ISCOM

The action of an adjuvant may be mediated through effects on: antigen targeting, antigen presentation or immune modulation.[65] The adjuvanting effects of ISCOMs appear to include all three of these actions.

### *Antigen targeting*

*In vitro* studies indicate that ISCOMs containing antigen (e.g. influenza virus envelope glycoproteins) adhere to and are taken up rapidly by peritoneal macrophages and other APC.[66] Uptake studies examining three populations of APC from mice, peritoneal cells (mainly macrophages and monocytes), dendritic cells from the spleen and naive B cells, demonstrated that there was a 30–70-fold higher internalization of biotinylated influenza ISCOMs than for influenza envelope proteins presented as protein micelles which were of a similar size.[67,68] The quantity of ISCOM-borne antigen found in peritoneal cells was approximately three to four times that found in dendritic cells or B cells and a high proportion (28%) of cell-associated antigen in these studies was detected in the cytosol.[67] The adherence of ISCOMs to cell and phagosomal membranes of peritoneal cells *in vitro*[66] presumably reflects the high affinity of Quil A/saponins for cholesterol and the powerful surfactant properties of Quil A. The higher uptake of ISCOMs by dendritic cells compared to protein micelles may be associated with the interaction of the ISCOM–saponin carbohydrates with a recently described molecule DEC-205,[69] which is capable of binding carbohydrate/glycoprotein ligands (although the natural ligands of the molecule have yet to be determined) via its 10 lectin-like domains. Targeting of DEC-205 has been shown to enhance endocytosis leading to the more efficient presentation of antigens to T cells.[69]

Several *in vivo* observations have been made with ISCOMs. Intraperitoneal injection of mice with labelled influenza antigen in ISCOMs resulted in relatively high levels of retained antigen in draining lymph nodes and in the spleen.[70] 'Inflammatory' responses that were observed included short-lived neutrophil accumulation in the peritoneal cavity that resolved in 24 h,[70] regional lymph node enlargement[71] with non-specific activation and cell proliferation demonstrated with splenocytes *in vitro* within 10 days of dosing.[72] Unlike oily adjuvants and aluminium salts, ISCOMs do not appear to function through a depot effect.[66,70] Cytokine release from mouse peritoneal cells pulsed with ISCOMs *in vitro* has been reported to include the 'inflammatory' cytokines such as IL-1, IL-6, TNF-α and GM-CSF.[67]

### *Antigen presentation*

Studies by van Binnendijk *et al.*[73] suggest that ISCOMs enter cells via endosomal vesicles as intact particles rather than fusing with the outer cell membranes or endosomal membranes and deliver antigen (e.g. measles virus fusion glycoprotein F) to the cytosol for degradation and loading of MHC class I in the endoplasmic reticulum for CTL induction.[67] As with liposomes, degradation of antigen must also occur within endosomes for MHC class II loading[74] because of the known potency of ISCOMs in the induction of DTH[45,75] and Th1 and Th2 (indicated respectively by IgG2a and IgG1) responses in mice.[76–78]

Earlier studies established that MHC class II (Ia antigen) expression is increased in peritoneal cell populations 24 h after intraperitoneal injection of ISCOMs[66] and the *in vitro* response to antigen in ISCOMs is accessory-cell dependent; that is, there is no direct stimulation of lymphocytes.[79] Moreover, ISCOM-borne antigen can be taken up by B cells *in vitro* for presentation to helper T cells.[80] The question of whether multimeric antigen is critical for adjuvant action is still open although limited data with one antigen preparation (ISCOMs containing biotinylated influenza viral proteins) suggest that ISCOMs with more than one epitope per molecule are more immunogenic.[52]

### *Immune modulation*

This third potential component of ISCOMs adjuvant activity is only now receiving attention through the analysis

of cytokine stimulation,[68] although earlier work had demonstrated that the Quil A component of ISCOMs was capable of immunomodulatory activity, including increasing responses to T cell-independent antigens.[81,82] As indicated above, 'inflammatory cytokines' including a large amount of IL-1 and IL-6 are induced by ISCOMs and ISCOM matrix from macrophages or monocytes. Furthermore, when primed murine T cells were incubated *in vitro* with either B cells, peritoneal cells or splenic dendritic cells and ISCOMs containing envelope glycoproteins from influenza, high levels of IFN-γ and IL-2 were induced when compared with the same cells incubated with protein micelles containing influenza proteins.[67,68] Inflammatory responses caused by the Quil A component of ISCOMs are unlikely to account entirely for the actions of ISCOMs as this can be abolished without the loss of adjuvant activity by adding liposomes containing cholesterol.[59]

The range of responses elicited by ISCOMs including class I-restricted CTL,[44,45,83] DTH,[45,75] Th1- and Th2-type antibodies[76–78,84] suggest that immunomodulatory influences will be difficult to dissect. Few reports have examined whether ISCOMs can direct immune responses to predominantly a Th1- or a Th2-type response *in vivo*. A recent study by Robinson *et al.*[78] using antigens from the nematode parasite *Trichinella spiralis* with ISCOMs, alum, Titermax or Freund's Complete adjuvant observed an essentially Th2-type response (mainly $IgG_1$) in mice regardless of the adjuvant used. It is possible that these parasite antigens and other antigens are potent immunomodulators in their own right and dominate the response profiles through Th subset induction independently of the adjuvant. Valensi *et al.*[85] examined the purified Quil A fraction QH-C when it was simply combined with an influenza vaccine and administered to naive mice (i.m. or s.c.). They found that QH-C induced high serum levels of IFN-γ and IL-2 and moderate levels of IL-5 and IL-6 cytokines and concluded that the response was associated with both Th1 and Th2 responses. Other adjuvants (Alum, MF-59 and MF-59 + MTP-PE; MF-59 + MPL) tested in this system induced essentially a Th2 type response with predominantly IL-5 and IL-6 induction. It would seem that the antigen targeting and presentation functions of ISCOMs may provide adequate explanations for their adjuvant activity, but current cytokine studies may well add to the emerging picture.

## Experimental vaccine results

A comprehensive review of *in vivo* studies with ISCOM-adjuvanted vaccine candidates has recently been published.[12] Table 3 summarizes a further 37 publications examining various vaccine candidates. Approximately 75% of the publications have focused on the use of ISCOMs with viral antigens. Since 1992, the major areas of interest have been Retroviridae (HIV-1, -2, SIV, FIV and FeLV) and Orthomyxoviridae (various strains of influenza). ISCOMs have been made with disrupted virus, from single or multiple recombinant proteins and synthetic peptides. I.m. and s.c. routes have been favoured with a few studies using the oral or intranasal routes for vaccination. Ben Ahmedia *et al.*[86] reported no protection from a homologous influenza challenge, in mice given a single low level dose of influenza–ISCOMs (0.25 μg HA) intranasally. This is consistent with earlier reports of intranasal vaccination against influenza where at least two doses of 1 μg HA[84] incorporated into ISCOMs was required for protection and three doses of 3.5 μg of a respiratory syncytial virus (RSV) protein was required for protection against an RSV challenge.[87] Results obtained with oral vaccination using ISCOM vaccines have been variable, with partial protection/clearance reported in some systems[48,88] but not in another[78] while peroral administration was superior to intragastric administration in a further study.[152] Results for i.m. and s.c. administration have generally been more consistent.

A broad range of assays, including specific antibody level determinations, virus neutralization, lymphocyte proliferation and DTH have been used to assess the *in vivo* effect of ISCOM vaccines. Importantly, a number of studies have also assessed the capacity of the ISCOM vaccines to induce protection from challenge (see Table 3). Protection against rotavirus infection in infant baboons was also achieved in a study by Snodgrass *et al.*[89] by injecting ISCOM matrix mixed with inactivated rotavirus into nursing baboons to induce serum and milk antibody which was then passed on to the infant baboons in the mothers breast milk.

## Characterization and quality control of ISCOM vaccines

For ISCOM-based vaccines to be licensed for use in man, the production process must be fully characterized and validated. This section will look briefly at how this process has been approached for experimental vaccines, for a licensed veterinary vaccine and work undertaken to allow testing of a human ISCOM-based influenza vaccine in a Phase I clinical trial.

### *Experimental vaccines*

Many of the experimental ISCOM-based vaccines reported have only been partially characterized, usually on the basis of morphology and a limited number of biochemical and immunological assays. Electron microscopy has been widely used to confirm the presence and size of the characteristic particles.[1,5,90] Size has also been determined by dynamic light scattering[3] and by density gradient centrifugation.[7,46,47,56,61,62] Assessment of the level of protein incorporation has been determined by Lowry[62] or Bradford[7] assay methods and more recently amino acid analysis following purification of ISCOMs by density gradient centrifugation.[3,7,62,90] When only one protein has been used, quantitative ELISA has also been employed.[49] SDS-PAGE and western blot analysis have been used extensively[7,35,38,49,56,62,90] to characterize the purity and type of proteins incorporated into ISCOMs. In many of these density gradient centrifugation has been

**Table 3** ISCOM used with various vaccine candidates in animal experimental models

| Vaccine | Antigen | Species (Strain) | Route | Dose (and No.) | Isotype, bioactivity | T cell tests | Protection | Reference |
|---|---|---|---|---|---|---|---|---|
| Viral | | | | | | | | |
| HIV-1 | gp120/160, p24–25 | Mouse (NMRI) | s.c | 1–10 μg × 3 | Ig, VN | NT | NT | Åkerblom *et al.*[40] |
| HIV-1 | gp160 | Rabbit (NZW) | i.m. | 25, 85 μg × 4 | Ig, VN | LP | NT | Levi *et al.*[96] |
| HIV-1 | gp120 | Rabbit (NZW) | i.m. | 10 μg × 3 | IgG, VN | NT | NT | Browning *et al.*[42] |
| HIV-2 | HIV-2, HIV-2 V3 peptides | Monkey (Macaque) | i.m. | 140 μg × 5 | IgG | NT | + + | Putkonen *et al.*[124] |
| HIV-2 | HIV-2 | Mouse (NMRI) | s.c | 0.5, 5 μg × 1 | IgG | NT | NT | Stickner *et al.*[99] |
| SIV | SIV + gp120/32 + p27 | Monkey (Rhesus) | ? | 1–5 μg + 2–25 μg | Ig | NT | +, + + +* | Osterhaus *et al.*[95] |
| SIV | SIV, gp120 | Monkey (Rhesus) | i.m. | 250 μg × 4 | Ig, VN | NT | −, + , + +* | DeVries *et al.*[98] |
| SIV | SIV | Monkey (Macaque) | s.c. | 100–500 μg × 3–4 | Ig, VN | NT | −, + + +* | Various[110] |
| FIV | p130 | Cat | s.c. | 10 μg × 2 | Ig | NT | NT | Rimmelzwaan *et al.*[41] |
| FeLV | *env* peptides | Mouse, rabbit, cat | i.m; s.c. | 50–100 μg × 2–3 | Ig, VN | NT | NT | Weijer *et al.*[51] |
| Influenza | A/Sichuan/2/87 | Mouse (BALB/c) | i.m. | 0.025–2.5 μg × 1 | IgG, IgA | NT | + + | Ben Ahmedia *et al.*[94] |
| Influenza | A/Equine/ | Horse | i.m. | 1–5 μg × 1–4 | Ig | NT | + | Mumford *et al.*[125,126] |
| Influenza | A/Turkey/Minnesota | Turkey | s.c. | 25–100 μg × 1 | Ig | LP, DTH | ± | Kodihalli *et al.*[127] |
| Influenza | A/Hong Kong/68 | Mouse (BALB/c) | i.m; i.n. | 0.25 μg × 1 | IgG, IgM, IgA | NT | + + , − | Ben Ahmedia *et al.*[77,86] |
| Influenza | A/Sichuan/2/87 | Mouse (BALB/c) | i.m., oral | 0.25 μg × 1–2 | IgG, IgA | NT | + + → + + + , + | Ghazi *et al.*[88] |
| Influenza | A/Asia/57 | Mouse (C3H/He) | Oral, s.c. | 100 μg × 3 | Ig, ADCC | CTL | ±, + + +** | Scheepers & Becht[152] |
| HSV-1 | HSV-1 (6 glycoproteins) | Mouse (N1H) | s.c. | 5 μg × 1 | IgG, VN | NT | + → + + | Ertuk *et al.*[128] |
| CDV | CDV | Seal | ? | ? × 3 | NT | NT | + | Visser *et al.*[129] |
| Cocksakie | Cocksakie virus | Mouse | ? | $10^6$ TCID × 2 | VN | NT | + + | Fohlman *et al.*[130] |
| EHV-1 | EHV-1 | Horse (Welsh pony) | i.m. | 25 μg × 3 | Ig | NT | ± | Hanant *et al.*[131] |
| Ps. rabies | Pseudo rabies envelope | Mouse, pig | s.c.; i.m. | ? × 1–3 | IgG | LP | + + + (mice) | Tulman *et al.*[132] |
| Ps. rabies | SHV-1 | Mouse, pig | s.c.; i.m. | 1.2 μg + 3,50 μg × 1 | IgG, VN | DTH | + + | Puentes *et al.*[97] |
| Measles | MVH peptides | Rabbit | i.m. | 10 μg × 3 | IgG | NT | NT | Pedersen *et al.*[50] |
| Bov. Ad. 3 | BAV-3 | Rabbit, cow | i.m. | 75 μg + 2, 200 μg × 2 | VN | NT | NT | York *et al.*[133] |
| Rotavirus | RRV, MMV18006 | Baboon | i.m. | ? × 3 | IgG,A,VN | NT | NT | Snodgrass *et al.*[89] |
| Bacterial | | | | | | | | |
| Salmonella | S. heidelberg/enteritidis | Turkey | s.c. | 2 mg | Ig | NT | + → + + | Charles *et al.*[134,135] |
| Tularaemia | F. tularensis | Mouse (BALB/c) | s.c | 3–10 μg | Ig | LP,CY | + | Golovliov *et al.*[136] |
| Parasitic | | | | | | | | |
| Malaria | *P. falciparum* $SC_{26}42$ | Rabbit, mouse | i.m., s.c. | 25 μg × 2, 0.1–20 μg × 2 | Ig | LP | NT | Lockyer *et al.*[137] |
| Malaria | *P. falciparum* 155/RESA | Rabbit, monkey | i.m. | 20 μg × 3 | Ig, MI | NT | NT | Sjölander *et al.*[138] |
| Malaria | *P. falciparum* CARP | Rabbit (NZW) | i.m. | 30 μg × 2 | MI | NT | NT | Sjölander *et al.*[139] |
| Tox. gondii | Extract, mainly p30/p22 | Mouse (Swiss Webster) | s.c. | 5 μg × 3 | IgG | DTH | + + , −* | Lunden *et al.*[140] |
| Coccidian | *Eimeria falciformis* p27 | Mouse (BALB/c) | Oral | 20 μg × 6 | IgG, IgA | LP | + | Kanzanji *et al.*[48] |
| Nematode | *Trichinella spiralis* ES Ag | Mouse (C57BL/10) | s.c., oral | 10, 45 μg × 2 | IgG, IgM | NT | ±, − | Robinson *et al.*[78] |
| Ticks | *Boophilus microplus* gut Ag | Cow | i.m. | 50–350 μg × 5 | IgG | NT | + | Jackson *et al.*[141] |

s.c., subcutaneous; i.m., intramuscular, i.n., intranasal; VN, virus neutralization; MI, inhibition of merazoite invasion; NT, not tested; LP, lymphocyte proliferation, CTL, cytotoxic lymphocyte; DTH, delayed hypersensitivity; *varying results depending on type of challenge; **varying results with oral or peroral dosing; CY, measurement of cytokine; ADCC, Abs dependent cellular cytotoxicity.

used as an initial step to separate incorporated proteins from unincorporated proteins. Appropriate controls are required to show that the protein, or protein micelles (without saponin) do not co-migrate with ISCOMs, which would make analysis impossible.[7] A clearer separation might be expected if only a single or a few proteins are used rather than a whole virus or bacterial lysate. While a simple method for quantitating the incorporation of molecules into ISCOMs has yet to be devised, new technologies such as capillary electrophoresis are being examined and show some early promise. Immunogold labelling using antibodies to identify specific proteins offers the potential of detecting antigens incorporated or associated with ISCOMs and while some laboratories have used this technique, no examples have been published to date. ELISA and radioimmunoprecipitation have been used to ensure that molecules incorporated into ISCOMs have retained their native conformation.[48,49,56,62,90] For specific molecules such as influenza HA and neuraminidase, other quantitative methods such as single radial immunodiffusion (SRID) and HA assay have also been used to assess antigen levels.[7]

Few workers have attempted to quantitate the other constituents of ISCOMs such as the Quil A, cholesterol and phospholipid content. Quil A levels have been detected by methods such as haemolysis in a rocket electrophoresis assay,[7] measuring the incorporation of $^3$H-labelled Quil A into the ISCOM-containing fraction[7] or by reversed phase HPLC.[3] Determination of the level of Quil A in the final vaccine is important because of the potential toxic effects of excessive doses in mice or rats. Kersten *et al.*[3] also quantitated cholesterol (by gas chromatography) and phospholipid (by phosphorus assay). Their conclusion was that providing the proportions of the starting materials (Quil A, phospholipid, cholesterol and protein) were carefully optimized, the ratios of the components when formed into ISCOMs were essentially unchanged. Furthermore there was no selective incorporation of any particular fractions of Quil A into ISCOMs. Other tests occasionally reported include: nucleic acid

content (which was very low in ISCOMs produced from DNA[4] and RNA[62] viruses), and haemolytic activity (which was low compared with free Quil A).[6,32] The toxicity of ISCOMs compared with free Quil A has been assessed by measuring the intracellular dehydrogenase activity and protein/DNA synthesis in cell cultures *in vitro*.[32] Following incorporation of a particular antigen or antigens into the ISCOMs, the immunogenicity of the resulting vaccine has been evaluated after immunization by measuring antibody responses (using ELISA, immunoprecipitation, HA inhibition, viral neutralization and western blot) with cell-mediated immune responses examined by DTH, CTL and cytokine assays (e.g. IFN-γ) and response to challenge (assessed by survival, weight loss, virus isolation and clearance kinetics).[4,12,13,91,92] A summary of the tests performed on recent vaccine candidates is shown in Table 3; similar studies have also been performed with model antigens such as ovalbumin, BSA and biotin.[43–46,52,93]

### *A licensed veterinary vaccine 'ISCOVAC FLU vet'*

A veterinary ISCOM-based influenza vaccine has been produced by Iscotec AB since 1988 to protect horses from equine influenza (an identical product is produced under licence by Mallinckrodt and sold for the same purpose in the UK and Ireland). A synopsis of the immunological and toxicological data for the Iscotec product is shown in Table 4. It is worth noting that none of the vaccine batches produced to date have been rejected due to unsatisfactory reactions during safety testing of the vaccine. In the field, adverse side effects caused by the ISCOVAC FLU vet. have been reported by Swedish veterinarians and customers to the National Veterinary Institute, Uppsala (SVA), or the Agency for Medical Products (AMP). From the time the product was launched approximately 1 million doses of the vaccine have been sold in Sweden with no reported adverse side effects registered at either the SVA or the AMP.

### *A Phase I human influenza–ISCOM vaccine*

The most careful characterization and quality control of an ISCOM-based vaccine to date has occurred during the development of an experimental human influenza–ISCOM vaccine by CSL Ltd (Melbourne, Australia). A full description of the testing is beyond the scope of this article and only a brief outline will be given here. Many of the tests developed to characterize the ISCOVAC FLU vet. have been used together with a number of other tests required for human vaccines. The vaccine is made by a slight variation of the dialysis method (see Table 2) with ultrafiltration used to remove detergent. The purified *Quillaia* saponin fractions QH-A and QH-C which are used in the ISCOM vaccine are tested for: colour, clarity, near infrared spectroscopy, reversed phase HPLC, monosaccharide analysis, solubility, pH, water content, residual solvents, presence of tannins or heavy metals, carbohydrate content, haemolytic activity, microbial load, abnormal toxicity, endotoxin levels, the ability to form ISCOMs in the correct QH-A:QH-C ratio and testing of the adjuvant activity of ISCOPREP 703. Formulated influenza–ISCOMs are examined for: sterility, pH, preservative content, endotoxin levels, MEGA-10 levels, total QH-A and QH-C (as well as the ratio), free QH-A and QH-C, haemolytic activity, particle size, abnormal toxicity, morphology (by electron microscopy), HA content (by SRID) and immunogenicity in mice.

## Comparisons between ISCOM and other vaccine adjuvants in experimental animals and man

A number of studies have compared a range of adjuvants in experimental animals (recent publications).[94–99] Various rankings have been obtained ranging from ISCOMs being superior to 'gold standards' like Freund's adjuvant[38] to equivalence with other adjuvants[97,98] or in some cases being inferior to other adjuvants.[99] These results should be considered carefully as much depends on

**Table 4** Synopsis of the components, recommendations and indications for ISCOVAC FLU vet. vaccine

| | | |
|---|---|---|
| Name of product | ISCOVAC Flu vet. | |
| Composition | 2 mL (1 dose) contains: | |
| | HA isolated from A/Eq 1/Prague/56 (H7N7) | ≥ 10 μg |
| | HA isolated from A/Eq 2/Borlange/91 (H3N8) | ≥ 20 μg |
| | *Quilluia* saponin (Quil A) derivative | ≤ 300 μg |
| | Cholesterol | ≤ 30 μg |
| | Phosphatidyl choline | ≤ 30 μg |
| | Neomycin | ≤ 50 μg |
| | PBS pH 7.4 | |
| Dosage form | Suspension for injection | |
| Target animal species | Horses | |
| Route of administration | Intramuscular injection | |
| Basic vaccination | Two vaccinations at 6–8 weeks interval from 6 months of age | |
| Re-vaccination | The third vaccination should be given 12 months after the second vaccination<br>Following the fourth vaccination, annual re-vaccinations are recommended | |
| Indications | Active immunization of healthy horses against equine influenza | |
| Interaction | For achievement of optimal immunizing effect, vaccination of horses less than 6 months of age is not recommended | |
| Storage | 2–8°C, vaccine is stable for up to 2 years from date of manufacture | |

the dose of antigen and adjuvant, the animal species and the criteria on which the assessment is being made. Few studies have extensively evaluated ISCOMs against a panel of adjuvants using a number of antigens, species, doses and, most importantly, read-out systems. Adjuvant comparisons because of their inherent complexity are usually done in a single species, with a single antigen, using either a small number of adjuvants assessed by many endpoints or with a larger number of adjuvants assessed by only a few endpoints. One such study evaluated ISCOMs, ISCOM matrix, $AlO_4$, MDPP (muramyl dipeptide) in a Tween 80 squalane emulsion and Freund's adjuvant in rabbits with a HIV-1 rgp160 protein and evaluated 11 immunological criteria based mainly on the nature of the antibody responses.[96] In this system ISCOMs ranked last behind ISCOM matrix, an MDP-containing emulsion and Freund's adjuvant for antibody induction but scored highest for T cell stimulation. In contrast, a comparison in mice and sheep with an influenza vaccine formulated in a series of oil in water preparations or ISCOMs (A Coulter unpubl. data) demonstrated that ISCOMs induced high antibody levels and protection against challenge (in mice) which was equivalent to the oil in water preparations, but caused less site reaction and had a higher level of CTL induction (Table 5).

Other factors that may affect the performance of ISCOMs *in vivo* are the method and level of incorporation of antigen and the dose of Quil A administered.[71] If too much Quil A is administered (especially in small animals) toxicity issues arise which may confuse the interpretation. In a recent publication, Stieneker *et al.*[99] injected NMRI mice with 5 μg of a split inactivated HIV-2 viral protein and ISCOMs containing approximately 50 μg of Quil A s.c. (a lethal dose of Quil A for this and some other mouse strains). To overcome the toxicity, the vaccine dose was reduced 10-fold which not surprisingly resulted in lower antibody responses. Better alternatives in this situation would have been to reduce the amount of Quil A and to reformulate the ISCOMs with the same amount of protein or to use the less toxic, purified *Quillaia* saponin fractions such as ISCOPREP 703.

ISCOM-based vaccines also have the ability to induce CTL, which may be important in the protection/recovery from some viral infections and in immunotherapy. The induction of CTL against specific antigens has been demonstrated for a number of ISCOM-based vaccines including influenza HA (R Macfarlan unpubl. data), influenza nucleoprotein,[13] the heat shock protein of mycobacteria,[100] HIV envelope proteins,[83] RSV[87] and for ovalbumin.[44,45,101] Other adjuvants have been shown to induce CTL with certain antigens under certain circumstances such as QS-21 with ovalbumin,[102] oil in water emulsions with ovalbumin[103] and HIV gp120,[103] $A1OH_3$ with influenza,[104] ovalbumin in monophosphoryl lipid A (MPL)-liposomes[105] and particular lipopeptides.[106] The ability of ISCOMs to induce CTL is a consistent finding which in the limited studies where they have been compared with other adjuvants were capable of achieving higher levels of CTL activity. Unfortunately, there are few reports that compare ability of different adjuvants to induce CTL and it is unclear at this stage whether any of these results are of predictive value in man.

The need to evaluate a number of candidate HIV vaccines provides an opportunity to test a range of the 'newer adjuvants' in humans. A large number of adjuvants are being tested for safety and efficacy for this purpose by the AIDS Evaluation Group (AVEG) in collaboration with the Center for Biological Evaluation and Research, FDA.[107,108] This group has developed a 'Rabbit Adjuvant Safety and Immunogenicity Testing Protocol'[109] as a standard way of testing candidate vaccine adjuvants prior to

Table 5 Comparison of oil in water adjuvants and ISCOMs formulated as an influenza vaccine

| Adjuvant formulation (given s.c.) | μg HA/dose | Median antibody titre U/mL | CTL induction | Dose site reactions | Response to challenge Weight loss (%) | Response to challenge Survival (%) |
|---|---|---|---|---|---|---|
| Virus alone | 1 | 79 | – | – | 26 | 70 |
| Virus alone | 0.1 | ND | ND | ND | 23 | 70 |
| ISCOMs* | 1 | 642 | + + | – | 0.9 | 100 |
| ISCOMs* | 0.1 | 308 | ND | ND | 7.8 | 100 |
| Squalene/L121† | 1 | 403 | ± | + + | 8 | 100 |
| Squalene/L121† | 0.1 | ND | ND | ND | 20 | 100 |
| Squalene‡ | 1 | 549 | ± | + | 14 | 100 |
| Squalene‡ | 0.1 | 386 | ND | ND | 17 | 90 |

Purified, disrupted A/PR-8/34 influenza virus was used but the dose was calculated on HA content. The median antibody titre was determined in BALB/c mice, $n = 10$. A value of 1 is equivalent to a titre of 100 in serum. Mice were bled 1 week after the second dose of vaccine. CTL induction was tested in BALB/c mice, $n = 3$. CTL activity was measured with spleen cells after restimulation with influenza virus infected cells then assayed for lysis of P815 influenza infected target cells. Dose site reactions were tested in sheep ($n = 6$) in a separate experiment with given 5 μg of HA. The response to challenge was tested in BALB/c mice, $n = 10$. An aerosol challenge was given to mice of approximately $1 \times 10^6$ $TCID_{50}$ per mouse of A/PR-8/34 influenza virus, 100% of unimmunized mice die with this challenge procedure. ND, not done; –, no response/reaction.

*Influenza ISCOMs were made with Quil A, mice were given approximately 10 μg of Quil A per dose, while sheep were given approximately 75 μg of Quil A per dose.

†Formulated to the same specifications as SAF-1 (see ref. 115).

‡Formulated to the same specifications as MF-59 (see ref. 112).

clinical trials. An initial series of 10 adjuvants that had been successfully tested in this system gave disappointing results when mixed with whole inactivated simian immunodeficiency virus (SIV) vaccines and injected into rhesus macaques.[107] No significant improvement in protection over alum-adjuvanted vaccines was reported following challenge with cell-free SIV. These adjuvants are currently being evaluated in Phase I human clinical trials (see AVEG 015, Table 6) even though only limited experimental animal data are available. Neither ISCOMs nor ISCOM matrix were included in this HIV clinical trial but they were included in a recent SIV trial in macaque monkeys in Europe.[110] These trials showed that an SIV ISCOM vaccine was capable of fully protecting monkeys (3/3) from challenge with homologous SIV grown in a human cell line; however, this result is complicated by the problem of immune responses to human HLA antigens incorporated into the ISCOMs. Partial (2/4) protection from challenge with cell-associated SIV grown in simian cell lines was achieved in monkeys with SIV-ISCOMs, but no protection was obtained in immunized monkeys when they were given an extracellular challenge of SIV grown in simian cells (no protection was seen with other adjuvants; Alum, MDP or RIBI).[110] In an earlier study 3/4 macaque monkeys were protected with an ISCOM-based HIV-2 vaccine (0/4 controls) from an i.v. challenge with 10 monkey infectious doses of a simian cell grown, cell-free HIV-2 for up to 18 months post-vaccination.[124]

Prior to the testing of an ISCOM-based influenza vaccine in man, animal toxicological and pharmacological testing was recently carried out by CSL (Melbourne, Australia) on ISCOPREP 703, ISCOMATRIX and on an influenza–ISCOM vaccine (both made with ISCOPREP 703). Based on the overall findings of these studies (which included acute and local toxicity, local tolerance, mutagenicity, pyrogenicity as well as a protocol based on the AVEG rabbit testing[109] outlined above) it was concluded that it was safe to administer influenza–ISCOM vaccine (made with ISCOPREP 703) to healthy adult volunteers by the i.m. route. Prior to the commencement of this clinical trial, a Phase I safety evaluation with ISCOMATRIX will be undertaken. The results of both of these clinical trials will be reported separately. Table 6 lists the adjuvants that are currently being evaluated with HIV vaccines as well as other recent human clinical trials using some of the 'newer adjuvants'. MF-59[111,112] is a microfluidized oil (squalene) in water emulsion currently being developed by the Chiron Corporation (USA) that has been used alone or augmented with an immunostimulant MTP-PE[113] (Ciba Geigy, Switzerland). QS-21 is a saponin from *Quillaia saponaria* Molina bark that is purified to near homogeneity and selected for high adjuvant activity and low toxicity that is being developed by Cambridge Biotech (USA).[23] DETOX developed by Ribi (USA) contains mycobacterial cell wall skeletons and MPL in an oil (squalane) emulsion.[114] SAF-1 developed by Syntex (USA) is a microfluidized oil in water emulsion similar to MF-59 but using squalane and the non-ionic block copolymer L-121.[115] In addition, combinations of these leading adjuvants such as MPL and QS-21 have also begun to be investigated in experimental animals[116] and QS-21 with alum in clinical trials.[117]

## Non-vaccine applications of ISCOM

As reflected in this review, the bulk of the applications of ISCOMs have been centred on their use in vaccines. A further application of ISCOM technology that has been suggested is as carriers of drugs such as amphotericin B[118,119] and other lipophilic drugs. A prerequisite for this work is the need for isolation or modification of a Quillaia saponin component that has little or no immunomodulatory activity and has an acceptable *in vivo* toxicity. QH-A has been suggested as a possible candidate but it remains to be seen whether this fraction is sufficiently inert in humans.[13] The term 'delpha' has been coined to describe these 'de-adjuvanted' ISCOMs. Further work is required to establish the value of this approach.

**Table 6** Completed and ongoing human clinical trials using some 'new generation' vaccine adjuvants

| Adjuvant | Vaccine | Antigen | Testing status | Reference |
|---|---|---|---|---|
| MULTIPLE* | HIV-1 | HIV-1 rgp120 | Phase I | 107, 117 |
| MF-59 + MTP-PE | HIV | rgp120 | Phase I (completed) | 142 |
| MF-59 + MTP-PE | Influenza | Whole virus | Phase I (completed) | 143 |
| QS-21 | Melanoma | GM2 ganglioside | Phase I (completed) | 144 |
| DETOX | Melanoma | Melanoma lysate | Phase I (completed) | 145 |
| DETOX | Melanoma | Melanoma proteins | Phase I (completed) | 146 |
| DETOX | Ovarian Ca | Disaccharide | Phase I (completed) | 147 |
| MPL + liposomes | Malaria | CSP protein | Phase I (completed) | 148 |
| MTP-PE | HIV-1 | env2-3 | Phase I (completed) | 149 |
| Virosomes | Influenza | Whole virus | Phase II (completed) | 150 |
| Virosomes | Hepatitis A | Whole virus | Phase I (completed) | 151 |
| ISCOM | Influenza | Whole virus | Phase I | Ongoing CSL† |
| Seppic ISA 720 | EBV | Peptide | Phase I | Ongoing QIMR‡ |
| Seppic ISA 720 | Malaria | Three recombinant proteins | Phase I | Ongoing Roche§ |

*AVEG 015 (Aids Evaluation Group): adjuvants being tested are; Alum, MF-59, MF-59 + MTP-PE, SAF/2, SAF/2 + MDP, liposomes-MPL-Alum, MPL-0.25% squalene. AVEG 016: adjuvants being tested are; Alum, QS-21 and Alum + QS21. †, Studies being carried by CSL, Australia; ‡, studies being carried out by Queensland Institute of Medical Research, Australia; §, studies being carried out by Roche, Switzerland and Saramane, Australia.

Another application of ISCOMs has been their use as antigens in immunoassays. Bjorkman *et al.*[120] used this system for the diagnosis of neosporosis in dogs. The incorporation capacity of ISCOMs was used to enrich for several relevant membrane antigens from the coccidian parasite *Neospira caninum*. These ISCOMs were then coated to ELISA plates and used as a screening assay with sera from normal and infected dogs. Compared with a crude lysate, there was a substantial improvement in the sensitivity and specificity of the ELISA when ISCOM 'purification' was used. These values correlated well with the results using the more laborious IFAT (indirect immunofluorescence test) and with clinical symptoms. Similar findings have been achieved using *Toxoplasma gondii*-ISCOMs for serological testing,[42] but it remains to be seen whether this application becomes more widely applied to other diagnostic testing.

## Unresolved issues

As outlined in this article, laboratory animal studies with ISCOMs have clearly demonstrated the capacity of this adjuvant preparation to elicit high serum antibody responses, prominent Th1 responses and strong CTL responses. The key unresolved issues centre around the mode of action of ISCOMs and their use in human vaccination. Fractionation of *Quillaia* saponin into various components has demonstrated a reduction in toxicity in animal testing. Whether this finding is translated into an acceptable level of systemic or local reactions in humans must await the completion of clinical trials. The dream of high adjuvant potency without any local or systemic reactions is probably just that, a dream.[121] However, the key issue is: 'Is there a dose of adjuvant in humans which provides adequate activity and acceptable reactivity?' ISCOMs prepared with different reversed phase HPLC saponin fractions or combinations of these fractions have been shown to have different immunological and toxicity profiles in animals[23,32] but the uniformity of action of any one ISCOM preparation has yet to be established in the genetically diverse human population.

Methods to consistently and predictably incorporate antigens, particularly high levels of hydrophilic proteins, into ISCOMs have yet to be devised. Moreover, details on the intracellular fate of ISCOM-delivered antigen into the various APC is unknown. Naturally, the important quantitative aspects of this type of study will take some time to elucidate. The oral route of administration (like many non-parenteral routes, for that matter) has not been explored systematically as yet. Great hopes are held for ISCOMs in immunotherapy and for the deviation of 'established', counter-productive or irrelevant immune responses in chronic infections. This has not yet been reduced to practice. Finally, the capacity of ISCOM-delivered antigen to induce a state of long-lived immunological memory (probably the key to prophylactic vaccination) has not been established definitively. Nevertheless as in so many aspects of ISCOM research the early signs are encouraging.

## Conclusions

It is now just over a decade since the characteristics of ISCOMs were first described. In this time they have grown from a novel immunomodulatory particle to one of the leading candidates for an improved adjuvant for use in human and animal viral vaccines. Clinical testing is in progress to determine whether modifications to the original methods and compounds have succeeded in reducing the toxicity of ISCOMs sufficiently while retaining their powerful adjuvant characteristics. The next 1–2 years will be a watershed for ISCOMs. This period will determine if they are to become part of the new vaccine technologies of the 21st century or whether they will be an interesting experimental system that is suitable for animals but will require further work for human applications.

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